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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q	A2	(11) International Publication Number: WO 99/15692 (43) International Publication Date: 1 April 1999 (01.04.99)
<p>(21) International Application Number: PCT/EP98/06009</p> <p>(22) International Filing Date: 21 September 1998 (21.09.98)</p> <p>(30) Priority Data: PI 970 4411 23 September 1997 (23.09.97) MY</p> <p>(71) Applicants (for all designated States except US): BAVARIAN NORDIC RESEARCH INSTITUTE A/S [DK/DK]; Naverland 2, DK-2600 Glostrup (DK). GSF-FORSCHUNGSZENTRUM FÜR UMWELT UND GESUNDHEIT GMBH [DE/DE]; Ingolstädter Landstrasse 1, D-85758 Neuherberg (DE). UNIVERSITI MALAYSIA SARAWAK [MY/MY]; Unimas Research Park, MY-94300 Kota Samarahan, Sarawak (MY). VENTURE TECHNOLOGIES SDN BHD [MY/MY]; Unimas Research Park, MY-94300 Kota Samarahan, Sarawak (MY).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): DREXLER, Ingo [DE/DE]; Hohenzollernstrasse 12, D-80796 München (DE). SUTTER, Gerd [DE/DE]; Germaniastrasse 15A, D-80802 München (DE). CARDOSA, Mary, Jane [MY/MY]; 96 Jalan Wan Abbul Rahman, Lorong 4, MY-93300 Kuchung, Sarawak (MY). HOOL, Tio, Phaik</p>		<p>[MY/MY]; 114 Lorong A 8A, Tamam Satria Jaya, BDC Stam, MY-93250 Kuchung, Sarawak (MY).</p> <p>(74) Agent: PIELKEN, Petra; Bavarian Nordic Research Institute GmbH, Fraunhoferstrasse 18b, D-82152 Martinsried (DE).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published Without international search report and to be republished upon receipt of that report.</p>
<p>(54) Title: DENGUE VIRUS ANTIGENS AND TREATMENT OF DENGUE FEVER</p>		
<p>(57) Abstract</p> <p>The present invention relates to monoclonal antibodies and antigen binding fragments specific for and recognising antigenic epitopes capable of eliciting antibodies which protect against infection by dengue viruses as well as the said antigenic epitopes and their use for vaccine purposes. The present invention also relates to recombinant vaccinia viruses and for example such which are derived from the Modified Vaccinia Ankara (MVA) encoding for and capable of expressing dengue virus antigen, and the use of the same recombinant viruses encoding dengue virus antigens in vaccines. In addition the invention also relates to pharmaceutical compositions comprising the above monoclonal antibodies, pharmaceutical compositions comprising dengue virus antigens, and pharmaceutical compositions comprising dengue virus antigen expression vectors.</p>		

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DENGUE VIRUS ANTIGENS AND TREATMENT OF DENGUE FEVER.

The present invention relates to monoclonal antibodies and antigen binding fragments specific for and recognising antigenic epitopes, including synthetic peptides, capable of eliciting antibodies which protect against infection by dengue viruses as well as the said antigenic epitopes and their use for vaccine purposes. The present invention also relates to recombinant vaccinia viruses and for example such which are derived from the Modified vaccinia virus Ankara (MVA) encoding for and capable of producing dengue virus antigens or antigenic epitopes recognised by said monoclonal antibodies, and the use of such recombinant viruses as vaccine. In addition the invention also relates to pharmaceutical compositions comprising the above monoclonal antibodies, pharmaceutical compositions comprising dengue virus antigens, and pharmaceutical compositions comprising expression vectors synthesising dengue virus antigen.

BACKGROUND OF THE INVENTION

Dengue viruses are divided into four antigenically related serotypes, called dengue virus serotypes 1, 2, 3, and 4. Complete or partial nucleotide sequences of the dengue 1, 2, 3, and 4 type virus have been published (Chamber, T.J., Hahn, C.S., Galler, R. And Rice, C.M. 1990. Annu. Rev. Microbiol., 44, 649, Zhao et al., 1986, Virology 155, 77-88).

Dengue virus, with its four serotypes Den-1 to Den-4, is the most important member of the Flavivirus genus with respect to infections of humans producing diseases that range from flu-like symptoms to severe or fatal illness, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue outbreaks continue to be a major public health problem in densely populated areas of the tropical and subtropical regions, where mosquito vectors are abundant. Therefore, there is a substantial need for the development of prophylactic vaccines. Ideally, a single vaccine would confer protection against several pathogens and would induce both cellular and humoral arms of immune response. Previous efforts to prepare live candidate dengue vaccines were mainly based on classical attenuation of dengue virus by serial passage in animals or in cultured cells of non-natural hosts. However, this approach has not been consistently successful in producing attenuated vaccine strains. Available

data indicate that recovery and protective immunity after dengue virus infection are correlated to the development of high titres of virus neutralising antibodies. However, this immunity is homotypic mediating resistance to the same virus serotype only. Moreover, individuals immune to one dengue virus serotype may be even at higher risk of developing severe dengue illness if reinfected with another serotype, a phenomenon described as immune enhancement or antibody mediated enhancement. To overcome these problems an ideal vaccine should therefore induce solid immunity against all four dengue virus serotypes without danger of potentiating more severe disease due to the phenomenon of immune enhancement.

Knowledge about the molecular biology of flaviviruses rapidly increased during the last decade, and led to the application of recombinant techniques for the production of new vaccine candidates. These approaches have included *E. coli* fusion proteins, baculo virus produced recombinant proteins, and live recombinant vaccinia viruses. Notably, the vaccinia approaches have given promising results. In mice, total protection against lethal challenge with dengue virus has been achieved after immunisation with recombinant vaccinia viruses expressing structural and/or non-structural genes of dengue virus.

The flavivirus genome consists of a single-stranded positive sense RNA molecule. The single encoded open reading frame (ORF) is translated into a polypeptide which is cleaved co- and post-translationally into at least 11 proteins. The order of proteins encoded in the ORF is 5'-C-preM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' (Venugopal, K., and Gould, E.A., 1994, Vaccine, Vol. 12, No. 11).

Previous results from vaccine trials in animal models indicated that immune responses to dengue structural preM and E proteins, or non-structural NS1 proteins were fully protective against a lethal challenge with homotypic dengue virus (Zhao et al. 1987 J. Virol. 61:4019; Bray et al. 1989 J. Virol. 63:2853; Falgout et al. 1990 J. Virol. 64:4356; Fonseca et al. 1994 Vaccine 12:279; Srivastava et al. 1995 Vaccine 13:1251).

The preM protein (18-19 kDa) is a precursor of the structural protein M, which is formed by cleavage and removal of the N-terminal (pre) segment, by a process presumed to be linked to maturation of the envelope glycoprotein and the development of virus infectivity.

The E glycoprotein (53-54 kDa) is an outer structural protein of the dengue virus. It exhibits a number of biological activities including receptor binding and membrane fusion, and is the target for neutralising antibodies and T-helper cells.

- 5 The E protein is a typical membrane glycoprotein with a C-terminal that spans the membrane.

- 10 The non-structural protein NS1 (39-41 kDa) is also modified by glycosylation. It is derived from the ORF by N-terminal signalase cleavage and C-terminal cleavage involving a protease. NS1 may be involved in the assembly and release of virions. It is found on the cell surface and in the culture medium of infected cells. During the course of infection, NS1 protein evokes a strong antibody response which protects the host against challenge with flaviviruses, presumably through a complement mediated pathway, although recently it has been
- 15 suggested that other mechanisms such as antibody-dependent cell cytotoxicity may also be responsible (Venugopal, K., & Gould, E.A., 1994, Vaccine, Vol. 12, No. 11).

- 20 A successful vaccine against the prototype yellow fever virus (YFV) has long been in use, as has been the less celebrated vaccines against tick borne encephalitis virus (TBEV) and Japanese encephalitis virus (JEV). These successes are unlikely to be easily reproduced for the dengue viruses which are the causative agents for a two important disease syndromes - classical dengue fever (DF) and dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). This
- 25 is because DHF/DSS has been shown to be associated with secondary immune responses to heterologous dengue virus serotypes which may be due to antibody dependent enhancement (ADE) of virus replication in host monocytes via an Fc receptor mediated pathway. The ADE phenomenon has been shown to be due to subneutralising concentrations of antibodies to the envelope glycoprotein (E).
- 30 For example in the presence of subneutralising concentrations of antibody bound to virus, Fc receptors or C3 complement receptors can mediate attachment and uptake in the mechanism termed antibody-dependent enhancement (ADE).

- 35 In the search for a safe and effective vaccine against DHF/DSS, it became necessary to address the question of the avoidance of ADE, and it was thus a welcome relief when Schlesinger's group reported that passive as well as active immunisation of mice against NS1 of Yellow Fever Virus (YFV) conferred solid protection against challenge with YFV (J Immunol, 135:2805-9, 1985; J Virol,

60:1153-55, 1986), a finding that was later confirmed for dengue virus NS1 as well (J Gen Virol, 68:853-57, 1987; J Gen Virol, 69:2102-7, 1988).

Since these early experiments however, flavivirus NS1 produced as various
5 recombinant proteins in several different expression systems has failed to live
up to the early promise of solid protection. Results have been conflicting and
although all recombinants generated have elicited a humoral response to NS1,
not all have been successful at protection in challenge experiments, with results
10 ranging from solid protection through partial protection through delayed death
and no protection at all.

Our studies into the role of antibodies to NS1 in patients with DF and DHF/DSS
carried out in collaboration with Suchitra Nimmannitya of Children's Hospital,
Bangkok, Thailand, show that there is a differential antibody response of DF and
15 DHF/DSS patients to different forms of dengue virus NS1. It has recently been
shown that the NS1 glycoprotein exists in the natural state as a dimer which on
heating degrades into the 46 kDa monomeric form which has been recognised in
conventional studies. Our data suggests that the vast majority of patients with
DHF (Grades I and II) and DSS (Grades III and IV) show a dominance of
20 antibodies to the dimer form of NS1 while those with DF are more likely to have
antibodies to both NS1 dimer and monomer. The absence of antibodies to NS1
monomer in most patients with more severe dengue illness may point to a role
for NS1 monomer in protection. This, however, appears to be untrue as we have
data on the kinetics of appearance of antibodies to NS1 dimer and monomer and
25 find that antibodies to NS1 monomer do appear early in the infection, may
disappear during the period of crisis and then reappear in convalescence. The
inverse relationship of antibodies to NS1 monomer to disease severity is a
critical factor to study in relation to vaccine design. There is a possibility that the
clearance of antibodies to NS1 monomer may be associated with the marked but
30 transient thrombocytopenia and hypovolemia often observed at the same time.
These differences in response to NS1 dimer and monomer in DF and DHF/DSS
patients may in fact be the key to the conflicting results obtained in the challenge
experiments performed after immunisation with recombinant proteins. Not all
these studies have addressed the question of dimerization of the recombinant
35 proteins generated, but on examination of the few studies which have addressed
this question, it is clear that those recombinant proteins which are expressed
primarily as dimers are more convincingly protective unlike those which are
primarily expressed as monomers or unstable dimers.

There is still the need for the development of a safe and an effective vaccine with a major goal in the prevention, and perhaps the treatment, of DF and DHF/DSS.

- 5 As mentioned above only approaches using recombinant vaccinia virus have so far given promising results. However, occurrence of rare adverse reactions to smallpox vaccination and the increased susceptibility of immunodeficient individuals has made further attenuation and improved safety a priority for human vaccines based on recombinant vaccinia virus.

10

Modified vaccinia virus Ankara (MVA), is a host range restricted and highly attenuated vaccinia virus strain, which is unable to multiply in human and most other mammalian cell lines tested. But since viral gene expression is unimpaired in non-permissive cells recombinant MVA viruses may be used as exceptionally
15 safe and efficient expression vectors.

The modified vaccinia virus Ankara (MVA) has been generated by long-term serial passages of the Ankara strain of vaccinia virus (CVA) on chicken embryo fibroblasts (for review see Mayr, A., Hochstein-Mintzel, V. and Stickl, H.: [1975]
20 Infection 3, 6-14; Swiss Patent No. 568, 392). The MVA virus was deposited in compliance with the requirements of the Budapest Treaty at CNCM (Institut Pasteur, Collection Nationale de Cultures de Microorganismes, 25, rue du Docteur Roux, 75724 Paris Cedex 15) on Dec. 15, 1987 under Depositary No. I-721. The MVA virus has been analyzed to determine alterations in the genome relative to
25 the wild type CVA strain. Six major deletions of genomic DNA compared with the wild type CVA (deletion I, II, III, IV, V, and VI) totalling 31,000 base pairs have been identified (Meyer, H., Sutter, G. and Mayr A. [1991] J. Gen. Virol. 72, 1031-1038). MVA is further distinguished by its great attenuation, that is to say by diminished virulence or infectivity while maintaining good immunogenicity.
30 When tested in a variety of animal models, MVA was proven to be avirulent even in immunosuppressed animals. More importantly, the excellent properties of the MVA strain have been demonstrated in extensive clinical trials (Mayr et al., Zbl. Bakt. Hyg. I, Abt. Org. B 167, 375-390 [1987], Stickl et al., Dtsch. med. Wschr. 99, 2386-2392 [1974]). During these studies in over 120,000 humans,
35 including high risk patients, no side effects were associated with the use of MVA vaccine. MVA replication in human cells was found to be blocked late in infection preventing the assembly to mature infectious virions. Nevertheless, MVA was able to express viral and recombinant genes at high levels even in

non-permissive cells and was proposed to serve as an efficient and exceptionally safe gene expression vector (Sutter, G. and Moss, B. [1992] Proc. Natl. Acad. Sci. USA 89, 10847-10851). Recently, novel vaccinia vector systems were established on the basis of MVA, having foreign DNA sequences inserted at the site of deletion III within the MVA genome or within the tk gene (Sutter, G. and Moss, B. [1995] Dev. Biol. Stand. Basel, Karger 84, 195-200 and US patent 5.185.146).

According to the present invention an effective prevention and therapy of DF, DHF and DSS has been made possible by the identification of dengue virus neutralising monoclonal antibodies which does not cause immune enhancement or is involved in antibody dependent enhancement, and by using one such monoclonal antibody, an antigenic discontinuous epitope in the dengue virus envelope protein which elicits neutralising antibodies without being involved in immune enhancement or antibody dependent enhancement has been identified. Further using this monoclonal antibody to select peptides from a random peptide library, peptides which may be used to elicit dengue neutralising antibodies and which do not cause immune enhancement or is involved in antibody enhancement have been identified. Further, according to the present invention, an antigen expression system for the *in vivo* or *ex vivo* production of a therapeutic agent against DF, DHF or DSS has been prepared.

OBJECTS OF THE INVENTION

It is an object of the present invention to provide monoclonal antibodies or antigen binding fragments that specifically bind to and identify dengue specific antigenic epitopes and especially dengue specific antigenic epitopes which are not involved in immune enhancement.

Another object of the present invention is to provide monoclonal antibodies or antigen binding fragments that specifically bind to and identify dengue specific antigenic epitopes and especially dengue specific antigenic epitopes which are not involved in immune enhancement for development of a treatment of and prevention of dengue infection, dengue haemorrhagic fever and dengue shock syndrome.

Still another object of the present invention is to provide antigenic epitopes and antigens which are not involved in immune enhancement and which are useful for vaccination against dengue virus.

- 5 A still further object of the present invention is to provide recombinant vaccinia virus based expression systems expressing dengue virus antigens or antigenic epitopes to provide an efficient and safe dengue virus vaccine for animals and humans.
- 10 Still one other object of the present invention is to provide heterologous and/or synthetic peptide sequences recognised by non-immune enhancing dengue virus neutralising monoclonal antibodies and DNA sequences (genes) which code for the same.

15

SUMMARY OF THE INVENTION

The present invention thus, inter alia, comprises the following, alone or in combination:

20

Dengue antigens or antigenic epitopes as well as heterologous and/or synthetic peptide sequences recognised by non-immune enhancing dengue virus neutralising monoclonal antibodies and DNA sequences (genes) which code for the same;

25

proteins, polypeptides, peptides and synthetic peptides including the amino acid sequence LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any other amino acid sequence or functional analogues or other equivalents thereof which will elicit anti-dengue antibodies not able to effect immune enhancement or

30 antibody dependent enhancement;

a monoclonal antibody that specifically binds to and identifies dengue specific antigenic epitopes and especially dengue specific antigenic epitopes which will elicit dengue neutralising antibodies not able to effect immune enhancement or

35 antibody dependent enhancement;

a monoclonal antibody as above, which is a monoclonal antibody of subclass IgG recognising proteins, polypeptides or peptides including the amino acid

sequences LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any amino acid sequence or functional analogues or other equivalents thereof and which will elicit dengue neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement;

5

a monoclonal antibody as above, which is a monoclonal antibody of subclass IgG1 recognising proteins, polypeptides or peptides including the amino acid sequences LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any amino acid sequence or functional analogues or other equivalents thereof and which will elicit dengue neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement;

10

a monoclonal antibody as above, which is a mouse monoclonal antibody of subclass IgG1 recognising proteins, polypeptides or peptides including the amino acid sequences LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any amino acid sequence or functional analogues or other equivalents thereof and which will elicit neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement;

15

a DNA construct encoding a dengue virus antigen as above, whereby the antigen is under the transcriptional control of a T7 RNA polymerase promoter;

20

a vaccine comprising as a first component a recombinant MVA carrying and capable of expressing T7 RNA polymerase and as further components one or more recombinant DNA vectors each carrying at least one dengue virus antigen or synthetic antigen not being involved in immune enhancement or antibody dependent enhancement and being recognised by a dengue virus neutralising antibody when the said antigen or synthetic antigen is under transcriptional control of a T7 RNA polymerase promoter;

25

30

a method for the treatment or prevention of a dengue virus infection comprising inoculating a living animal body, including a human, in need thereof with the first and further components of a vaccine, as above, either simultaneously or with a timelag but using the same inoculation site;

35

a recombinant MVA containing and capable of expressing one or more DNA sequences encoding dengue virus antigens not able to effect immune enhancement or antibody dependent enhancement;

a recombinant MVA as above, containing and capable of expressing DNA sequences encoding antigens from any of the four dengue virus serotypes (type 1, 2, 3 and 4) not able to effect immune enhancement or antibody dependent
5 enhancement;

A recombinant MVA as anyone above wherein the dengue virus antigen includes the amino acid sequence LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any amino acid sequence or a functional analogue or other
10 equivalent thereof which will elicit neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement;

a recombinant MVA as above, containing and capable of expressing DNA sequences encoding peptides, polypeptides or proteins being recognised by
15 monoclonal antibodies specific to the peptides, polypeptides and/or proteins including the amino acid sequence LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any amino acid sequence or a functional or other equivalent thereof which will elicit dengue neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement;

a recombinant MVA as above, wherein the DNA sequences encoding antigen is under transcriptional control of a vaccinia specific promoter selected from the group of vaccinia virus early/late promoter P7.5, vaccinia virus synthetic promoter or any other equivalent promoter;

a vaccine containing at least one recombinant MVA as anyone above and a pharmaceutically acceptable carrier or diluent; or

a method for the treatment or prevention of dengue virus infection comprising
30 administering to a living animal body, including a human, in need thereof a therapeutically effective amount of a recombinant MVA as anyone above, or a vaccine as above.

35

THE PRESENT INVENTION

Monoclonal antibodies generated to be specifically directed against Dengue virus polypeptides are essential to identify and to target Dengue virus-specific

epitopes which enables the rational design of novel prophylactic, therapeutic, and diagnostic tools for the control of human Dengue virus infection.

5 According to the invention, using panels of dengue virus specific neutralising monoclonal antibodies, the locations in the envelope glycoprotein of dengue virus of a series of protective epitopes are identified. In addition, such neutralising antibodies are screened for immune enhancing properties in order to determine that even at sub-neutralising concentrations the antibodies do not cause antibody dependent enhancement.

10 In one embodiment of the present invention, a specific mouse monoclonal antibody identified as MAS1 which specifically recognises peptides, polypeptides and proteins which contain a sequence functioning as a *neutralising* but *non immune enhancing* epitope for the elicitation of protective immune responses against dengue virus infection is provided.

15 The monoclonal antibody MAS1 can be prepared by anyone skilled in the art and as described generally by Kohler, G. & Milstein, C. (1975, Nature, 256: 495-497) and Harlow, E. & Lane, D. (1988, Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratories). The monoclonal antibody MAS1 according to one preferred embodiment of the present invention, is a mouse monoclonal antibody of subclass IgG1 which has the property of being able to neutralise dengue virus without causing antibody dependent enhancement in Fc receptor bearing cells at any dilution tested. An epitope defined by this monoclonal antibody MAS1 has the advantage of being an epitope which is neutralizable but not enhanceable and therefore represents an epitope which would be safe and efficacious as a vaccine preparation, being able to elicit protective antibodies which will not cause immune enhancement at any concentration.

20 25 According to the invention, neutralizing monoclonal antibodies which are not immune enhancing are used in the screening of different bacteriophage display libraries, peptide libraries etc. to identify peptide sequences recognised by the said non-immune enhancing dengue virus neutralising monoclonal antibodies.

30 35 The said specific monoclonal antibody MAS1 has been found to recognise peptides, polypeptides or proteins which contain a sequence which functions as a protective but not enhancing epitope for the elicitation of a protective immune response against dengue virus infection. Such sequences may be derived from

the authentic dengue virus proteins or glycoproteins themselves or from synthetic peptides or other mimetic peptides or polypeptides, including but not limited to anti-idiotypic antibodies and their derivatives as well as DNA sequences encoding any of the above-mentioned molecules.

- 5 The monoclonal antibody MAS1 has been found to recognise polypeptides including the amino acids sequence LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL.
- 10 The monoclonal antibody MAS1 is further characterised as being a mouse monoclonal antibody within the IgG1 family recognising the amino acid sequence LPWYNHS and furthermore by the fact it recognises a discontinuous antigenic epitope within the E gene of dengue 2.
- 15 Further, according to the present invention, DNA sequences (genes) which code for dengue antigens or antigenic epitopes as well as heterologous and/or synthetic peptide sequences recognised by non-immune enhancing dengue virus neutralising monoclonal antibodies, and especially by the MAS1, capable of eliciting immune responses to dengue virus are thus provided for the treatment
- 20 of and prevention of dengue virus infection. Such sequences are optionally introduced, with the aid of DNA recombination techniques, into the genome of viral expression systems including for example the MVA vector. When the DNA sequence encoding the dengue antigen or antigenic epitope is integrated at a site in the viral DNA which is non-essential for the life cycle of the virus, e.g. one of
- 25 the above mentioned deletions, the newly produced recombinant MVA will be infectious, that is to say able to infect foreign cells and it will express the integrated DNA sequence. The recombinant viruses and the recombinant MVA according to the invention will be useful as extremely safe live vaccines for the treatment or prophylactics of dengue infection.
- 30 The recombinant MVA vaccinia viruses can be prepared as set out hereinafter.
- For homologous recombination of the wild type MVA and a heterologous nucleotide acid sequence in a virus infected cell a DNA-construct containing
- 35 sequences flanking the left and the right side of a naturally occurring deletion, e.g. deletion II or III, within the MVA genome (Altenburger, W., Suter, C.P. and Altenburger J. (1989) Arch. Virol. 105, 15-27) was constructed.

A DNA-construct which contains a DNA-sequence which codes for a dengue antigen or antigenic epitope flanked by MVA DNA sequences adjacent to a naturally occurring deletion, e.g. deletion II or III, within the MVA genome, is introduced into cells infected with MVA, to allow homologous recombination.

5 The DNA-construct to be inserted can be linear or circular. A circular DNA is preferred, especially a plasmid.

The DNA sequence encoding antigenic epitopes is inserted between the sequences flanking the naturally occurring deletion.

10

For the expression of a DNA sequence encoding antigenic epitopes, it is necessary for regulatory sequences, which are required for the transcription of the DNA sequence, to be present on the DNA. Such regulatory sequences (called promoters) are known to those skilled in the art, and include for example those of the vaccinia 11 kDa gene as are described in EP-A-198, 328, those of the 7.5 kDa gene (EP-A-110, 385), the vaccinia specific synthetic promoter sP (Sutter et al., 1994, Vaccine 12, 1032-1040) or those of the bacteriophage T7 Polymerase.

15

The DNA-construct can be introduced into the MVA infected cells by transfection, for example by means of calcium phosphate precipitation (Graham et al., Virol. 52, 456-467 [1973]; Wigler et al., Cell 777-785 [1979] by means of electroporation (Neumann et al., EMBO J. 1, 841-845 [1982]), by micro-injection (Graessmann et al., Meth. Enzymology 101, 482-492 (1983)), by means of liposomes (Straubinger et al., Methods in Enzymology 101, 512-527 (1983)), by means of spheroplasts (Schaffner, Proc. Natl. Acad. Sci. USA 77, 2163-2167 (1980)) or by other methods known to those skilled in the art. Transfection by means of calcium phosphate precipitation is preferred.

20

25

Once the DNA-construct has been introduced into the eukaryotic cell and the foreign DNA has recombined with the viral DNA, it is possible to isolate the desired recombinant vaccinia virus in a manner known per se, preferably with the aid of a marker (compare Nakano et al., Proc. Natl. Acad. Sci. USA 79, 1593-1596 [1982], Franke et al., Mol. Cell. Biol. 1918-1924 [1985], Chakrabarti et al., Mol. Cell. Biol. 3403-3409 [1985], Fathi et al., Virology 97-105 [1986]).

30

35

The present invention therefore also relates to recombinant MVA which contains at least one DNA sequence which codes for dengue virus antigen or antigenic

epitopes and relates further to vaccines containing such viruses in a physiologically acceptable form.

5 The recombinant MVA according to the invention contain and are capable of expressing one or more DNA sequences encoding dengue virus antigens or antigenic epitopes capable of eliciting a protective immune response against dengue viruses.

10 In a preferred embodiment according to the invention the dengue virus antigen includes the amino acid sequence LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL or any functional analogue or other equivalents thereof.

The invention therefore in a preferred embodiment relates to a viral expression vector, and preferably a pox virus based expression system and especially a
15 MVA based expression system coding for peptides, polypeptides or proteins including the amino acid sequences LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL including for example the following synthetic expression sequence containing one start codon and two stop codons for translation: 5'- CAG CAG CCC GGG ATG CTT CCG TGG TAT AAT CAT TCT GGT CCT -3' and 5'-CAG
20 CAG CCC GGG CTA TTA GCC CTC ATA GTT AGC GTA ACG -3'

The invention also relates to vaccines comprising recombinant MVA according to the invention containing and capable of expressing one or more DNA
25 sequences encoding dengue virus antigens.

In one such embodiment, a vaccine according to the invention comprises a recombinant MVA encoding dengue virus type 1 antigen; recombinant MVA encoding dengue virus type 2 antigen; recombinant MVA encoding dengue virus
30 type 3 antigen; recombinant MVA encoding dengue virus type 4 antigen; and/or includes the amino acid sequence LPWYNHS APWYTHP, TPWYL, LPWYPSP, TPWYTHL or any functional analogue or other equivalents thereof.

The invention further relates to vaccines comprising recombinant MVA
35 according to the invention containing and capable of expressing one or more DNA sequences encoding peptides or polypeptides comprising antigenic epitopes recognised by monoclonal antibody MAS1 which elicit protective immune responses against dengue virus infection.

In one embodiment therefore, the vaccine according to the invention comprises a peptide, polypeptide or protein which contains an epitope recognised by the said monoclonal antibody MAS1. The epitope can exist within an amino acid
5 sequence derived from random peptide libraries and identified by the said monoclonal antibody MAS1, or the epitope can exist within an anti-idiotypic antibody generated against said monoclonal antibody MAS1, or can exist within polypeptides and proteins encoded by the envelope genes of dengue viruses and includes but is not limited to nucleic acid sequences which encode for the epitope
10 described by monoclonal antibody MAS1.

In another embodiment a DNA vectors carrying DNA sequences encoding dengue antigens under transcriptional control of a T7 RNA polymerase promoter were provided.
15

In still another embodiment of the invention recombinant MVA encoding T7 RNA polymerase (MVA-T7pol) is used in combination with DNA vectors carrying DNA sequences encoding dengue antigens under transcriptional control of a T7 RNA polymerase promoter.
20

According to this embodiment, a coding sequence of a given dengue antigen capable of binding to a monoclonal antibody not being involve in immune enhancement is cloned under control of a T7 RNA polymerase promoter preferably in a plasmid vector and the resulting DNA construct is amplified and
25 purified using standard laboratory procedures. Secondly, the vector DNA is inoculated simultaneously or with appropriate timelags together with MVA-T7pol. An appropriate timelag allows a cell to take up the inoculated DNA vector, before MVA infection takes place. The mode of administration, the dose and number of administration can be optimised by one skilled in the art. At the
30 site of inoculation the recombinant gene of interest is expressed transiently in cells containing both the vector DNA and MVA-T7pol and the corresponding antigen is presented to the host immune system stimulating an antigen-specific immune response.

35 The recombinant MVA according to the invention can also carry a marker gene. Said marker genes are preferably selected from the group consisting of marker genes which codes for proteins such as β -galactosidase, neomycin, alcohol dehydrogenase, luciferase, puromycin, hypoxanthine phosphoribosyl transferase

(HPRT), hygromycin, and green or blue fluorescent proteins. Such a marker gene can either facilitate the purification of recombinant MVA or be used as a independent antigenic marker to control status of immune response after a antigen specific vaccination.

5

The invention also relates to methods for the preparation of such recombinant MVA and vaccines, and to the use of these vaccines for the prophylactics or treatment of infections caused by dengue virus.

- 10 For the preparation of vaccines, the recombinant MVA according to the invention are converted into a physiologically acceptable form. This can be done based on the experience in the preparation of MVA vaccines used for vaccination against smallpox (as described by Stickl, H. et al. [1974] Dtsch. med. Wschr. 99, 2386-2392). Typically, about 10^6 - 10^8 particles of the recombinant MVA are
- 15 freeze-dried in 100 ml of phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule. The lyophilisate can contain extenders (such as mannitol, dextran, sugar, glycine, lactose or polyvinylpyrrolidone) or other aids (such as antioxidants, stabilisers, etc.) suitable for parenteral administration. The glass ampoule is then sealed and
- 20 can be stored, preferably at temperatures below -20°C , for several months.

- For vaccination or therapy the lyophilisate can be dissolved in 0.1 to 0.5 ml of an aqueous solution, preferably physiological saline, and administered either parenterally, for example by intramuscular inoculation or locally. Vaccines or
- 25 therapeutics according to the invention are preferably injected intramuscularly (Mayr, A. et al. [1978] Zbl. Bakt. Hyg., I. Abt. Orig. B 167, 375-390). The mode of administration, the dose and the number of administrations can be optimised by those skilled in the art in a known manner. It is expedient where appropriate to administer the vaccine several times over a lengthy period in order to obtain
- 30 appropriate immune responses against the foreign antigen.

- The detailed examples which follow are intended to contribute to a better understanding of the present invention. However, the examples are not intended to confine the invention to the subject-matter of the examples. Rather, the
- 35 invention may be worked according to numerous equivalent or similar procedures all being well known in the art and all of such equivalent or similar procedures to obtain and effect the steps of the present invention, will be appreciated as such by any person of average skill in the art, and should be

considered part of and comprised by the present invention and application and the invention is therefore only to be limited by the full scope of the appended claims. Also it will be appreciated by the person of an average skill in the art, that the MVA vector used herein only constitute an example of an efficient gene
5 expression system and the said MVA vector may be conveniently substituted or exchanged for any suitable gene expression system.

EXAMPLE 1

10 Growing of dengue 2 virus

A continuous mosquito cell line, C6/36 was grown to confluency in a 25 mm² tissue culture flask in Leibovitz 15 medium supplemented with 3% fetal calf serum (FCS), 10% tryptose phosphate broth, penicillin (100 units/ml), streptomycin (100 mg/ml). The medium was then replaced with 4.5 ml of
15 maintenance medium (Leibovitz 15 medium supplemented with 1% FCS, 10% tryptose phosphate broth, and antibiotics in the concentrations described above. Half a ml of Dengue infectious virus stock culture supernatant was added and the flask was rocked gently at room temperature overnight. The flask was then maintained in a stationary position in an incubator set at 28°C for 4 to 5 days
20 until some signs of a cytopathic effect were seen. The medium was then changed to 5 ml of fresh maintenance medium and the cells were incubated at 28°C until syncytia formation occurred. When the monolayer was almost completely fused, the supernatant was harvested for infectious virus. The supernatant was clarified by centrifugation for 10 minutes at 4 °C at 5000 rpm in a Beckman Avanti J25
25 using a JS7.5 rotor. The clarified supernatant was the Dengue infectious virus stock and was used for seeding new cultures as well as for all Dengue virus plaque assays including neutralization assays and immune enhancement assays.

EXAMPLE 2

30 Plaque Titration of Dengue 2 Virus

The Dengue infectious virus stock was titrated in a plaque assay in the following manner. A confluent monolayer of PS Clone D cells were trypsinized and resuspended at 2×10^5 cells/ml maintenance medium (composition as described
35 in Example 1) and 0.5 ml was dispensed in each well of a 24 well tissue culture cluster plate. Ten fold serial dilutions of Dengue infectious virus stock were prepared in maintenance medium and 100 µl of these dilutions or maintenance medium was added in duplicate to each well containing PS Clone D cells in

suspension. The plate was gently tapped to mix the contents of each well and the plate then was incubated at 37 °C for 4 hours until the cells had adhered to the plate. The cells were then overlaid with 0.6 ml of maintenance medium containing 1.5% carboxymethylcellulose, and incubated at 37 °C for 4 to 5 days until plaques were visible under an inverted microscope. The medium was then tipped out into a container containing 5% chlorox and the monolayers gently washed with phosphate buffered saline, pH 7.4. The monolayers were then stained for 15 minutes with naphthalene black and washed with water. The plates were allowed to airdry and the plaques counted.

Virus dilution	1/10	1/100	1/1000	1/10 000	1/100 000
Mean number of plaques	too many to count	167	18	4	0

EXAMPLE 3

Assay for Neutralisation of Dengue 2 Virus

A dilution of Dengue virus infectious stock which would give approximately 30-40 plaques per well based on the above titration was then selected and prepared by dilution into maintenance medium. The dilution used in this example was 1/500. Hybridoma culture supernatant containing the monoclonal antibody to be tested was diluted in four fold serial dilutions in maintenance medium. 100 µl of undiluted hybridoma supernatant or dilutions were dispensed into 24 well tissue cluster plates in triplicate. Control supernatant from hybridomas secreting irrelevant monoclonal antibody (for example to Hepatitis B virus) was also dispensed in triplicate and these wells containing monoclonal antibody or control were then seeded with 100 µl of virus dilution prepared previously.

The plate was tapped gently to mix the contents and incubated at 37 °C for one hour.

A confluent monolayer of PS Clone D cells was resuspended at 3×10^5 cells/ml maintenance medium as described above and dispensed at 0.3 ml per well

containing the virus-antibody incubation mixtures or controls. Cells were also added to triplicate wells containing no incubation mixtures. The plate was incubated at 37 °C for 4 hours until the cells had adhered to the plastic and 0.5 ml of maintenance medium containing 1.5% carboxymethylcellulose was overlaid.

5 The plate was incubated at 37 °C for 4 to 5 days until plaques were visible using an inverted microscope. The monolayers were then washed and stained with naphthlene black as described above and plaques counted. Neutralization of Dengue virus was deemed to have occurred when the mean number of plaques in wells containing antibody was at least 50% less than in the control wells

10 containing irrelevant antibody or no antibody.

Monoclonal antibody dilution	undiluted	1/4	1/16	1/64	irrelevant antibody
Mean number of plaques	0	4	10	39	36

EXAMPLE 4

15 Assay for Immune Enhancement of Dengue 2 Virus

A dilution of Dengue virus infectious stock which would give approximately 30-40 plaques per 100 µl based on the above titration was then selected and prepared by dilution into maintenance medium. Hybridoma culture supernatant

20 containing the monoclonal antibody to be tested was diluted in ten fold serial dilutions in maintenance medium. Similar dilutions were prepared of irrelevant hybridoma supernatants as controls. Duplicate wells containing 100 µl of maintenance medium was used as the negative control. 100 µl of undiluted hybridoma supernatant or dilutions were dispensed into 5 ml round bottomed

25 cell culture tubes. 100 µl of the Dengue virus dilution was added to each tube. The mixture was gently mixed and incubated at 37 °C for 1 hour.

A confluent monolayer of P388D1 cells were resuspended at 5×10^5 cells/ml into Leibovitz 15 maintenance medium and dispensed at 0.5 ml per tube. The tubes

30 were incubated at 37 °C overnight. The cells were then washed by centrifugation for 10 minutes at 800 rpm at room temperature in a Beckman Avanti J25 using a JS7.5 rotor. The supernatant was discarded and 1 ml of fresh maintenance

medium was added per tube. The tubes were centrifuged again as described and replenished with 1 ml of fresh maintenance medium. The cells were resuspended and incubated at 37 °C for 5 days. On the 5th day, cells were pelleted by centrifugation for 10 minutes at 1000 rpm at 4 °C in a Beckman Avanti J25 using a JS7.5 rotor, and the supernatants were titrated on PS Clone D cells as described in Example 2.

Immune enhancement or antibody dependent enhancement is deemed to have occurred when the number of plaque forming units (pfu) per ml of culture was double or greater than that of the negative controls containing no antibody.

Monoclonal antibody dilution	undiluted	1/10	1/100	1/1000	negative control
pfu/ml	850	1240	420	15	23

EXAMPLE 5

15 Assay for Non Immune enhancing Properties of Neutralising Antibodies

Ten fold serial dilutions of the monoclonal antibodies (MAS 1, MAS 2, MAS 3) to be tested were prepared in maintenance medium and used simultaneously in an assay for neutralising antibodies and in an assay for immune enhancement as essentially described in Examples 3 and 4. The antibody was considered to be neutralizing *and* non-enhancing only if there was no enhancing activity as defined in example 4 even at dilutions of monoclonal antibody when neutralizing activity was no longer seen.

MONOCLONAL ANTIBODY	UNDILUTED		1/10		1/100	
	NEUT	ADE	NEUT	ADE	NEUT	ADE
MAS 1	+	-	+	-	-	-
MAS 2	-	+	-	+	-	-
MAS 3	+	-	-	+	-	+

EXAMPLE 6

MAS1 Preparation and Purification

A Balb/c mouse was immunised through the ip route with Dengue antigen prepared as follows. C6/36 mosquito cell monolayers were inoculated with Dengue 2 virus as described in Example 1. When massive syncytia formation had occurred, the monolayer was washed once with phosphate buffered saline and lysed with 2 ml of isotonic buffer (1.5 mM magnesium chloride, 5 mM potassium chloride, 10 mM HEPES) containing 1% Nonidet P 40 after gentle rocking at room temperature for 10 minutes, the lysate was harvested and spun for 5 minutes in a microfuge. The supernatant was loaded into a single well comb of a 12% SDS PAGE gel (Biorad Mini Protean II) and run at 100 Volts until the dye front reached the end of the gel. Bands corresponding to Dengue Envelope glycoprotein and pre Membrane protein were excised from the gel, placed in a dialysis bag, electro-eluted in a minimal volume and used as the immunogen.

The immunised mouse was boosted through the ip route after two weeks with 0.5 ml infectious dengue virus culture supernatant prepared as described in Example 1 and left to rest for a week before the spleen was removed and spleen cells harvested. Cell fusion was attained by the method as essentially described by Kohler & Milstein, *Nature* 256, 495-497 (1975) and Kohler et al., *Eur.J.Immunol.* 6, 292-295 (1976) and clones were screened for anti Dengue activity by a dot enzyme immunoassay (Cardosa, M.J. & Tio, P.H., 1991. Dot enzyme immunoassay: Alternative diagnostic aid for dengue fever and dengue haemorrhagic fever, *Bulletin of the World Health Organisation*, 69, 741-745.) and by a mouse immunoglobulin capture ELISA using 96 well ELISA plates coated with anti-mouse immunoglobulin incubated with tissue culture supernatants overnight at 4 °C. After washing with phosphate buffered saline containing 0.05% Tween 20, the immunoglobulin bound was identified using dengue virus prepared as described in Example 1 followed by an HRP conjugated anti dengue convalescent human serum (Innis, B.L. Nisalak, A., Nimmannitya, S., et al., 1989. An enzyme-linked immunosorbent assay to characterise dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg* 40:418-27) Positive clones were expanded and recloned by limiting dilution twice.

The hybridoma which yielded MAS 1 was grown to large quantities in 75 mm² tissue culture flasks and supernatants harvested every 2 or 3 days. The supernatant was clarified by centrifugation at 3000 rpm at 4 °C for 15 minutes in

a Beckman Avanti J25 using a JS7.5 rotor, and the clarified supernatant was pushed through a 0.22 micron filter before purification through a HiTrap Protein G column (Pharmacia Biotech, Product Code No. 17-040-01) according to the manufacturer's instructions. Fractions containing protein were pooled and
 5 checked for specific activity in a dot enzyme immunoassay (Cardosa & Tio, 1991).

EXAMPLE 7

Screening of Bacteriophage Display Library

10

The affinity purified monoclonal antibody MAS 1 was used in panning experiments to select peptide ligands from a phage display peptide library purchased from New England Biolabs (Catalog number 8100). The experiment was performed according to the instructions of the manufacturer using MAS 1 as
 15 the panning antibody bound to the plastic surface of an microtitre ELISA plate. Three pannings were performed and phage selected were purified and DNA sequences of the heptapeptide displayed in each clone was determined. The DNA sequence was translated and consensus peptides were determined using the software Lasergene. The following are the consensus sequences obtained by
 20 comparison of amino acid sequences.

ALIGNMENT USING IOTUN HEIN METHOD

First line indicates the consensus sequence. Clones (characterised by a number)
 25 from all three pannings are listed in the first row.

	L	P	W	Y	P	S	P
1-1	L	S	S	S	I	T	E
1-2	H	A	A	S	V	L	C
1-3	G	A	P	S	P	S	E
1-4	I	L	V	G	H	Q	P
1-5	C	L	S	W	S	P	E
1-6	M	S	M	W	I	T	E
1-7	L	P	N	T	Y	Y	P
1-8	T	N	H	M	I	P	E
1-9	V	T	S	P	E	S	S
2-1	Q	L	V	P	W	S	H

2-2	S	L	W	P	P	H	L
2-3	T	P	W	Y	Q	A	R
2-4	F	P	G	W	M	N	H
2-5	S	N	K	Y	L	Q	L
2-6	L	P	W	Y	R	V	S
2-7	T	T	P	P	T	F	M
2-8	T	Q	S	S	T	L	Q
2-9	D	F	T	P	P	R	L
3-1	L	L	R	S	P	S	P
3-2	H	I	W	Y	T	P	W
3-3	L	P	W	A	P	H	K
3-4	A	E	P	V	A	M	L
3-5	H	Q	S	N	L	T	L
3-6	P	F	A	Y	N	S	Q
3-7	A	F	A	P	W	S	H
3-8	T	L	Q	P	W	W	H
3-9	T	N	S	R	P	P	S
3-10	Q	A	T	S	S	P	P
3-11	S	H	T	H	T	H	
3-12	I	P	W	F	O	Y	A
3-13	G	H	G	I	G	P	Q
3-14	G	P	W	Y	T	H	R
3-15	R	P	P	G	Y	I	P
3-16	A	Q	W	Y	Q	T	P
3-17	L	P	W	Y	N	H	S
3-18	W	A	H	A	N	R	Q

5

ALIGNMENT USING CLUSTAL METHOD

First line indicates the consensus sequence. Clones (characterised by a number) from the second and third pannings only are listed in the first row.

10

	-	-	T	P	W	Y	L	-	-
2-1	Q	L	V	P	W	-	-	S	H
2-2	S	L	W	P	P	H	L	-	-
2-3	-	-	T	P	W	Y	Q	A	R
2-4	-	-	F	P	G	W	M	N	H
2-5	S	-	-	N	K	Y	L	Q	L
2-6	-	-	L	P	W	Y	R	V	S
2-7	T	T	P	P	T	F	M	-	-
2-8	T	-	-	Q	S	S	T	L	Q
2-9	D	F	T	P	P	R	L	-	-

ALIGNMENT USING CLUSTAL METHOD

First line indicates the consensus sequence. Clones (characterised by a number)
 5 from the third panning are listed in the first row.

	A	P	W	Y	T	H	P
3-1	L	L	R	S	P	S	P
3-2	H	I	W	Y	T	P	W
3-3	L	P	W	A	P	H	K
3-4	A	E	P	V	A	M	L
3-5	H	Q	S	N	L	T	L
3-6	P	F	A	Y	N	S	Q
3-7	A	F	A	P	W	S	H
3-8	T	L	Q	P	W	W	H
3-9	T	N	S	R	P	P	S
3-10	Q	A	T	S	S	P	P
3-11	S	H	T	H	T	H	
3-12	I	P	W	F	O	Y	A
3-13	G	H	G	I	G	P	Q
3-14	G	P	W	Y	T	H	R
3-15	R	P	P	G	Y	I	P
3-16	A	Q	W	Y	Q	T	P
3-17	L	P	W	Y	N	H	S
3-18	W	A	H	A	N	R	Q

ALIGNMENT USING IOTUNHEIN METHOD

First line indicates the consensus sequence. Clones (characterised by a number) from the third panning are listed in the first row.

	A	P	W	Y	T	H	P
3-1	L	L	R	S	P	S	P
3-2	H	I	W	Y	T	P	W
3-3	L	P	W	A	P	H	K
3-4	A	E	P	V	A	M	L
3-5	H	Q	S	N	L	T	L
3-6	P	F	A	Y	N	S	Q
3-7	A	F	A	P	W	S	H
3-8	T	L	Q	P	W	W	H
3-9	T	N	S	R	P	P	S
3-10	Q	A	T	S	S	P	P
3-11	S	H	T	H	T	H	
3-12	I	P	W	F	O	Y	A
3-13	G	H	G	I	G	P	Q
3-14	G	P	W	Y	T	H	R
3-15	R	P	P	G	Y	I	P
3-16	A	Q	W	Y	Q	T	P
3-17	L	P	W	Y	N	H	S
3-18	W	A	H	A	N	R	Q

5

ALIGNMENT USING CLUSTAL METHOD

10 First line indicates the consensus sequence. Clones (characterised by a number) from the second and third pannings are listed in the first row.

	T	P	W	Y	T	H	L
2-1	Q	L	V	P	W	S	H
2-2	S	L	W	P	P	H	L
2-3	T	P	W	Y	Q	A	R
2-4	F	P	G	W	M	N	H
2-5	S	N	K	Y	L	Q	L

2-6	L	P	W	Y	R	V	S
2-7	T	T	P	P	T	F	M
2-8	T	Q	S	S	T	L	Q
2-9	D	F	T	P	P	R	L
3-1	L	L	R	S	P	S	P
3-2	H	I	W	Y	T	P	W
3-3	L	P	W	A	P	H	K
3-4	A	E	P	V	A	M	L
3-5	H	Q	S	N	L	T	L
3-6	P	F	A	Y	N	S	Q
3-7	A	F	A	P	W	S	H
3-8	T	L	Q	P	W	W	H
3-9	T	N	S	R	P	P	S
3-10	Q	A	T	S	S	P	P
3-11	S	H	T	H	T	H	
3-12	I	P	W	F	O	Y	A
3-13	G	H	G	I	G	P	Q
3-14	G	P	W	Y	T	H	R
3-15	R	P	P	G	Y	I	P
3-16	A	Q	W	Y	Q	T	P
3-17	L	P	W	Y	N	H	S
3-18	W	A	H	A	N	R	Q

EXAMPLE 8

Growing of the MVA virus

5

10

The MVA virus is a highly attenuated vaccinia virus derived from the vaccinia virus strain Ankara (CVA) by long-term serial passages on primary chicken embryo fibroblast (CEF) cultures. For a general review of the history of the production, the properties and the use of MVA strain, reference may be made to the summary published by Mayr et al. in *Infection* 3, 6-14 [1975]. Due to the attenuation in CEF, the MVA virus replicates to high titers in this avian host cell. In mammalian cells, however, MVA is severely growth restricted, and typical plaque formation by the virus is not detectable. Therefore, MVA virus was grown on CEF cells. To prepare CEF cells, 11-days old embryos were isolated

from incubated chicken eggs, the extremities are removed, and the embryos are minced and dissociated in a solution composed of 0.25% trypsin at 37 °C for 20 minutes. The resulting cell suspension was filtered and cells were sedimented by centrifugation at 2000 rpm in a Sorvall RC-3B centrifuge at room temperature for 5 minutes, resuspended in 10 volumes of medium A (MEM Eagle, for example obtainable from Life Technologies GmbH, Eggenstein, Germany), and sedimented again by centrifugation at 2000 rpm in a Sorvall RC-3B centrifuge at room temperature for 5 minutes. The cell pellet was reconstituted in medium A containing 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 mg/ml) and 2 mM glutamine to obtain a cell suspension containing 500 000 cells/ml. CEF cells obtained in this way were spread on cell culture dishes. They were left to grow in medium A in a 6% CO₂ atmosphere with 95% humidity at 37°C for 1-2 days, depending on the desired cell density, and were used for infection either directly or after one further cell passage. A detailed description of the preparation of primary cultures can be found in the book by R.I. Freshney, "Culture of animal cell", Alan R. Liss Verlag, New York [1983] Chapter 11, page 99 et seq.

MVA viruses were used for infection as follows. CEF cells were cultured in 175 cm² cell culture bottles. At 90-100% confluence, the medium was removed and the cells were incubated for one hour with an MVA virus suspension (0.01 infectious units (IU) per cell, 0.02 ml/cm²) in medium A. Then more medium A was added (0.2 ml/cm²) and the bottles were incubated at 37°C for 2-3 days (until about 90% of the cells show cytopathogenic effect). Crude virus stocks were prepared by scraping cell monolayers into the medium and pelleting the cell material by centrifugation at 3000 rpm in a Sorvall RC-3B centrifuge at 4°C for 5 minutes. The crude virus preparation was stored at -20°C before further processing (e.g. virus purification).

EXAMPLE 9

Cloning of MVA virus

To generate homogeneous stock virus preparations MVA virus obtained from Prof. Anton Mayr was cloned by limiting dilution during three consecutive passages in CEF cultured on 96-well tissue culture plates. The MVA clone F6 was selected and amplified in CEF to obtain working stocks of virus that served as starting material for the generation of recombinant MVA viruses described in this patent application as well as for the generation of recombinant MVA viruses described previously (Sutter, G. and Moss, B. [1992] Proc. Natl. Acad. Sci. USA 89,

10847-10851; Sutter, G., Wyatt, L., Foley, P., Bennink, J. and Moss, B. [1994] Vaccine 12, 1032-1040; Hirsch, V., Fuerst, T., Sutter, G., Carroll, M., Yang, L., Goldstein, S., Piatak, M., Elkins, W., Alvord, G., Montefiori, D., Moss, B. and Lifson, J. [1996] J. Virol. 70, 3741-3752).

5

EXAMPLE 10

Purification of the viruses

The purification steps undertaken to obtain a virus preparation which was as pure as possible and free from components specific to the host cell were similar to those described by (Joklik, Virology 18, 9-18 [1962]). Crude virus stocks which had been stored at -20°C. were thawed and suspended once in PBS (10-20 times the volume of the sediment), and the suspension was centrifuged as above. The new sediment was suspended in 10 times the volume of Tris buffer 1 (10 mM Tris-HCl pH 9.0), and the suspension was briefly treated with ultrasound (Labsonic L, B. Braun Biotech International, Melsungen Germany; 2x 10 seconds at 60 watts and room temperature) in order to further disintegrate cell debris and to liberate the virus particles from the cellular material. The cell nuclei and the larger cell debris were removed in the subsequent brief centrifugation of the suspension (Sorvall GSA rotor obtainable from DuPont Co., D-6353 Bad Nauheim, FRG; 3 minutes at 3000 rpm and 10°C.). The sediment was once again suspended in Tris buffer 1, treated with ultrasound and centrifuged, as described above. The collected supernatants containing the free virus particles were combined and layered over a cushion of 10 ml of 36% sucrose in 10 mM Tris-HCl, pH 9.0, and centrifuged in a Beckman SW 27/SW 28 rotor for 80 minutes with 13,500 rpm at 4°C. The supernatant was discarded, and the sediment containing the virus particles was taken up in 10 ml of 1 mM Tris-HCl, pH 9.0, homogenised by brief treatment with ultrasound (2x 10 seconds at room temperature, apparatus as described above), and applied to a 20-40% sucrose gradient (sucrose in 1 mM Tris-HCl, pH 9.0) for further purification. The gradient was centrifuged in Beckmann SW41 rotor at 13,000 rpm for 50 minutes at 4°C. After centrifugation, discrete bands containing virus particles were harvested by pipetting after aspirating volume above band. The obtained sucrose solution was diluted with three volumes PBS and the virus particles were sedimented again by centrifugation (Beckmann SW 27/28, 60 minutes at 13,500 rpm, 4°C). The pellet, which now consisted mostly of pure virus particles, was resuspended in PBS and equilibrated to virus concentrations corresponding on average to 1-5x

10⁹ IU/ml. The purified virus stock solution was stored at -80°C and used either directly or diluted with PBS for subsequent experiments.

EXAMPLE 11

5 Construction of vector plasmids

To allow the generation of recombinant MVA viruses novel vector plasmids were constructed. Insertion of foreign genes into the MVA genome was targeted precisely to the site of the naturally occurring deletion II in the MVA genome.

10 Sequences of MVA DNA flanking the site of a 2500 bp deletion in the HindIII N fragment of the MVA genome (Altenburger, W., Suter, C.P. and Altenburger, J. [1989], J. Arch. Virol. 105, 15-27) were amplified by PCR and cloned into the multiple cloning site of plasmid pUC18. The primers for the left 600 bp DNA flank were 5'-CAG CAG GGT ACC CTC ATC GTA CAG GAC GTT CTC-3' and 5'-

15 CAG CAG CCC GGG TAT TCG ATG ATT ATT TTT AAC AAA ATA ACA-3' (sites for restriction enzymes KpnI and SmaI are underlined). The primers for the right 550 bp DNA flank were 5'-CAG CAG CTG CAG GAA TCA TCC ATT CCA CTG AAT AGC-3' and 5'-CAG CAG GCA TGC CGA CGA ACA AGG AAC TGT AGC AGA-3' (sites for restriction enzymes PstI and SphI are underlined). Between

20 these flanks of MVA DNA inserted in pUC18, the *E. coli* lacZ gene under control of the vaccinia virus late promoter P11 (prepared by restriction digest from pIII LZ, Sutter, G. and Moss, B. [1992] PNAS USA 89, 10847-10851) was inserted, using the BamHI site, to generate the MVA insertion vector pUCII LZ [Figure 1]. In the following, a 289 bp fragment containing the vaccinia virus early-late promoter

25 P7.5 together with a SmaI site for cloning (prepared by restriction digest with EcoRI and XbaI from the plasmid vector pSC11 [Chakrabarti et al. 1985, Molecular and Cellular Biology 5, 3403-3409]) was inserted into the SmaI site of pUCII LZ to give the MVA vector pUC II LZ P7.5. To construct a vector plasmid that allows isolation of recombinant MVA viruses via transient synthesis of the

30 reporter enzyme b-galactosidase a 330 bp DNA fragment from the 3' end of the *E. coli* LacZ open reading frame was amplified by PCR (primers were 5'-CAG CAG GTC GAC CCC GAC CGC CTT ACT GCC GCC-3' and 5'-GGG GGG CTG CAG ATG GTA GCG ACC GGC GCT CAG-3') and cloned into the Sall and PstI sites of pUC II LZ P7.5 to obtain the MVA vector pUC II LZdel P7.5. Using the

35 SmaI site, this vector plasmid can be used to insert DNA sequences encoding a foreign gene under transcriptional control of the vaccinia virus promoter P7.5 into the MVA genome. After the desired recombinant virus has been isolated by screening for expression of β -galactosidase activity further propagation of the

recombinant virus leads to the self-deletion of the reengineered P11-LacZ expression cassette by homologous recombination.

EXAMPLE 12

5 Construction of recombinant MVA virus expressing a minigene for induction of Dengue virus-specific immune responses

A 147 bp minigene encoding a polypeptide including the N-terminal amino acids LPWYNHS and containing one start codon and two stop codons for

- 10 translation was prepared by PCR from cloned DNA of a phage M13 library using synthetic oligonucleotides 5'- CAG CAG CCC GGG ATG CTT CCG TGG TAT AAT CAT TCT GGT CCT -3' and 5'- CAG CAG CCC GGG CTA TTA GCC CTC ATA GTT AGC GTA ACG -3' and cloned into the SmaI restriction site of the MVA vector plasmid pUCII LZdel P7.5 to create the plasmid pUCII LZdel P7.5-DENmini1 carrying the minigene under transcriptional control of the vaccinia virus early/late promoter P7.5.

The minigene under control of the vaccinia virus early/late promoter P7.5 was inserted into deletion II within the MVA using homologous recombination. CEF cells infected with MVA at a multiplicity of 0.05 TCID₅₀ per cell were transfected

- 20 with DNA of plasmid pUC II LZdel P7.5-DENmini as described previously (Sutter, G, Wyatt, L., Foley, P., Bennink, J. and Moss, B. [1994] Vaccine 12, 1032-1040). Recombinant MVA viruses containing the Dengue virus cDNA sequence and transiently co-expressing the *E. coli* LacZ marker gene were selected by consecutive rounds of plaque purification in CEF cells stained with 5-bromo-4-chloro-3-indolyl β -D-galactoside (300 mg/ml). In the following, recombinant MVA viruses containing the E sequence and having deleted the LacZ marker gene were isolated by three additional consecutive rounds of plaque purification screening for non-staining viral foci in CEF cells in the presence of 5-bromo-4-chloro-3-indolyl β -D-galactoside (300 mg/ml).
- 30 Subsequently, recombinant viruses were amplified by infection of CEF monolayers.

CLAIMS:

- (1) Dengue antigens or antigenic epitopes as well as heterologous and/or synthetic peptide sequences recognised by non-immune enhancing dengue virus neutralising monoclonal antibodies and DNA sequences (genes) which code for the same.
- (2) Proteins, polypeptides, peptides and synthetic peptides including the amino acid sequence LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any other amino acid sequence or functional analogues or other equivalents thereof which will elicit anti-dengue antibodies not able to effect immune enhancement or antibody dependent enhancement.
- (3) A monoclonal antibody that specifically binds to and identifies dengue specific antigenic epitopes and especially dengue specific antigenic epitopes which will elicit dengue neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement.
- (4) A monoclonal antibody according to claim 3, which is a monoclonal antibody of subclass IgG recognising proteins, polypeptides or peptides including the amino acid sequences LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any amino acid sequence or functional analogues or other equivalents thereof and which will elicit dengue neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement.
- (5) A monoclonal antibody according to claim 4, which is a monoclonal antibody of subclass IgG1 recognising proteins, polypeptides or peptides including the amino acid sequences LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any amino acid sequence or functional analogues or other equivalents thereof and which will elicit dengue neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement.
- (6) A monoclonal antibody according to claim 3, which is a mouse monoclonal antibody of subclass IgG1 recognising proteins, polypeptides or peptides including the amino acid sequences LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any amino acid sequence or functional analogues or

other equivalents thereof and which will elicit dengue neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement.

- 5 (7) A DNA construct encoding a dengue virus antigen according to anyone of claims 1 to 3, whereby the antigen is under the transcriptional control of a T7 RNA polymerase promoter.
- 10 (8) A vaccine comprising as a first component a recombinant MVA carrying and capable of expressing T7 RNA polymerase and as further components one or more recombinant DNA vectors each carrying at least one dengue virus antigen or synthetic antigen not being involved in immune enhancement or antibody dependent enhancement and being recognised by a dengue virus neutralising antibody when the said antigen or synthetic antigen is under transcriptional control of a T7 RNA polymerase promoter.
- 15 (9) A method for the treatment or prevention of a dengue virus infection comprising inoculating a living animal body, including a human, in need thereof with the first and further components of a vaccine according to claim 8 either simultaneously or with a timelag but using the same inoculation site.
- 20 (10) A recombinant MVA containing and capable of expressing one or more DNA sequences encoding dengue virus antigens not able to effect immune enhancement or antibody dependent enhancement.
- 25 (11) A recombinant MVA according to claim 10 containing and capable of expressing DNA sequences encoding antigens from any of the four dengue virus serotypes (type 1, 2, 3 and 4) not able to effect immune enhancement or antibody dependent enhancement.
- 30 (12) A recombinant MVA according to claims 10 to 11 wherein the dengue virus antigen includes the amino acid sequence LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any amino acid sequence or a functional analogue or other equivalent thereof which will elicit dengue neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement.
- 35

- (13) A recombinant MVA according to claims 10 to 12 containing and capable of expressing DNA sequences encoding peptides, polypeptides or proteins being recognised by monoclonal antibodies specific to the peptides, polypeptides and/or proteins including the amino acid sequence LPWYNHS, APWYTHP, TPWYL, LPWYPSP, TPWYTHL, any amino acid sequence or a functional or other equivalent thereof and which will elicit dengue neutralising antibodies not able to effect immune enhancement or antibody dependent enhancement.
- 10 (14) A recombinant MVA according to claims 10 to 13 wherein the DNA sequences encoding antigen is under transcriptional control of a vaccinia specific promoter selected from the group of vaccinia virus early/late promoter P7.5, vaccinia virus synthetic promoter or any other equivalent promoter.
- 15 (15) A vaccine containing at least one recombinant MVA according to anyone of claims 10 to 14 and a pharmaceutically acceptable carrier or diluent.
- 20 (16) A method for the treatment or prevention of dengue virus infection comprising administering to a living animal body, including a human, in need thereof a therapeutically effective amount of a recombinant MVA according to claims 10 to 14, or a vaccine according to claim 15.